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Uncoupled human flavin-containing monooxygenase 3 can release superoxide radical in addition to hydrogen peroxide

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Abstract

Human flavin-containing monooxygenase 3 (hFMO3) is a drug-metabolizing enzyme capable of performing N- or S-oxidation using the C4a-hydroperoxy intermediate. In this work, we employ both wild type hFMO3 as well as an active site polymorphic variant (N61S) to unravel the uncoupling reactions in the catalytic cycle of this enzyme. We demonstrate that in addition to H$_2$O$_2$ this enzyme also produces superoxide anion radicals as its uncoupling products. The level of uncoupling was found to vary between 50 and 70% (WT) and 90 to 98% (N61S) for incubations with NADPH and benzydamine over a period of 5 or 20 minutes, respectively. For the first time, we were able to follow the production of the superoxide radical in hFMO3, which was found to account for 13-18% of the total uncoupling of this human enzyme. Moreover, measurements in the presence or absence of the substrate show that the substrate lowers the level of uncoupling only related to the H$_2$O$_2$ and not the superoxide radical. This is consistent with the entry point of the substrate in this enzyme’s catalytic cycle.

These findings highlight the importance of the involvement of hFMO3 in the production of radicals in the endoplasmic reticulum, as well as the relevance of single-nucleotide polymorphism leading to deleterious effects of oxidative stress.

Keywords: flavin-containing monooxygenase, hydrogen peroxide, superoxide radical, polymorphic variant, N61S, uncoupling.
1. Introduction

Human flavin-containing monooxygenase 3 (hFMO3) is a drug metabolizing enzyme expressed at high levels in the human liver [1-3]. Human FMO3 is the major human enzyme able to perform the monooxygenation of trimethylamine (TMA) yielding trimethylamine N-oxide (TMAO) [4]. Impaired metabolism of its main substrate TMA is known to cause trimethylaminuria, a genetic disease in which affected individuals present high levels of TMA in both sweat and urine leading to a displeasing body odor [5,6]. The disease is caused by mutations of the hFMO3 gene that cause an altered activity of the enzyme and have direct consequences on the oxidation of TMA. One such mutation, N61S, leads to a polymorphic variant known to cause trimethylaminuria [7]. On the other hand, the product of the reaction, TMAO, was recently found to be a risk factor for cardiovascular disease [8-10].

Previous characterization of pig liver FMO has shown how FMO is reduced by NADPH and upon binding of O$_2$ it can form a stable long-lived C4a-hydroperoxyflavin intermediate [11,12]. This species reacts very well with soft nucleophiles performing a monooxygenase reaction, followed by the formation of water and the release of the oxidized NADP$^+$ cofactor [11,12]. Nevertheless, recent work carried out with purified human FMO1, FMO2, FMO3 and FMO5 have demonstrated how the human enzyme does not seem to form a long-lived intermediate and that the detachment of oxygen species from peroxylavin might lead to uncoupling [13-16]. The uncoupling reaction, which is defined as the wastage of electrons and oxygen without oxidation of the substrate, can lead to the formation of reactive oxygen species (ROS) such as the superoxide radical and/or hydrogen peroxide. Earlier studies performed with pig FMO have highlighted the formation of superoxide anion radical at the rate of about 4% the total NADPH oxidized [17]. Formation of hydrogen peroxide, up to 41% of the total NADPH oxidized, has also been demonstrated in earlier work with purified rabbit lung FMO [18]. However, more
recently the human FMOs 1, 2 and 3 were found to release only H$_2$O$_2$ after reduction by NADPH with no superoxide radical being observed [16].

In this work, since we had previously purified hFMO3 [19] and its polymorphic N61S variant [13], we set out to measure the uncoupling products of these enzymes in order to clarify whether the human enzyme also produces the superoxide radical or only hydrogen peroxide. To do so, we tested the ability of the enzyme to use the electrons provided by NADPH to perform the monooxygenation reaction, by quantifying both the amount of ROS (hydrogen peroxide and superoxide radical) and product formed. The N61S polymorphic variant was selected due to its poor binding affinity for NADP$^+$ leading to an acceleration of the C4a-hydroperoxyFAD intermediate decay [13], resulting in higher uncoupling.

2. Material and Methods

2.1 Cloning, expression and purification

The pJL2-hFMO3 plasmid constructed previously [19, 20] was used with QuikChange® site-directed mutagenesis kit (Stratagene) to produce N61S hFMO3 [13]. Human FMO3 gene containing a C-terminal poly-histidine tag was heterologously expressed in E. coli cells and purified as described previously [21]. The protein was stored at -80 ºC in 50 mM KPi pH 7.4, 20% glycerol with 1 mM EDTA. Protein purity and concentration was determined spectroscopically as described previously [13, 22].

2.2 NADPH oxidation, hydrogen peroxide and superoxide detection

All the reactions were carried out in the dark, in triplicates, in 96-well plates using a plate reader equipped with a uv-vis absorbance detector using 1.6 µM hFMO3, 160 µM NADPH and 0 or 300 µM benzydamine in 50 mM KPi pH 7.4. The optical pathlength was 0.45 cm. The plate reader automatically takes into account the optical pathlength on the basis of the volume used
in the 96-well plates. For NADPH consumption reactions, mixtures were incubated at 37 °C.
NADPH signal at 340 nm was monitored in time using 6220 M⁻¹ cm⁻¹ as extinction coefficient.
Preliminary controls were performed to establish reaction conditions that do not lead to undesired production of hydrogen peroxide by NADPH autoxidation. The controls are shown in Figure S1 where incubations of AMPLEX RED kit are reported with or without NADPH. Results indicate that under our experimental conditions there is no detectable contribution to hydrogen peroxide formation by NADPH. Basal NADPH consumption in the absence of the substrate was also tested (Figure S2).
Potential interference of benzydamine with the Amplex Red assay was evaluated by comparing identical assay conditions in the presence or absence of benzydamine. Figure S3 shows that there is no interference with the assay.
For every assay aimed at the quantification of hydrogen peroxide the amount of hydrogen peroxide in the unknown sample was determined using a calibration curve prepared with fresh hydrogen peroxide and run in parallel with the unknown samples.
For H₂O₂ detection 50 μL of reaction mixture was withdrawn from a well at each time point and mixed in an eppendorf containing 50 μL of cold acetonitrile to terminate the reaction, centrifuged at 14000 rpm for 5 minutes and subjected to HPLC separation. The remaining 50 μL of reaction mixtures were incubated with AMPLEX RED [16, 23, 24] and H₂O₂ formation was quantified at 571 nm by recording the spectrum of resorufin after 1 minute of incubation for each time point. In the absence of benzydamine all the steps were identical, but the reactions were not subjected to HPLC. In order to assess if benzydamine can be oxidized non-enzymatically by H₂O₂ a reaction control was performed by incubating 20 μM of with benzydamine at 37°C for 1 hour. The concentration of was chosen to reproduce the highest amount of present in the reaction mixture produced by the enzyme.
For superoxide radical detection, in order to exclude that the signal of cytochrome c reduction is not due to superoxide, 2 control reactions were performed. In the first control, the cytochrome c reduction experiment was performed in the presence of SOD using 10 µM of cytochrome c. Figure S4, clearly shows that at this cytochrome c concentration there is no direct cytochrome c reduction by the enzyme. In the second control, cytochrome c reduction was performed in the absence/presence of SOD using 50 µM of cytochrome c. The data presented in Figure S5, demonstrate that at this higher cytochrome c concentration there is a contribution of hFMO3 to direct cytochrome c reduction. Therefore, only the SOD inhibited part of the signal can be used to measure superoxide. The amount of superoxide matches what was previously found using 10 µM cytochrome c that is the optimal concentration to test cytochrome c reduction without the interference of direct reduction from hFMO3 by cytochrome c.

To calculate the amount of superoxide produced by hFMO3 an extinction coefficient of 2.1x10^4 M^{-1}cm^{-1} was used which is the difference in the extinction coefficients between the reduced and oxidized forms and it was used because it takes into account only the amount of superoxide that is actually formed during the reaction. All analyses were carried out using 10 µM horse heart cytochrome c (Sigma).

2.3 HPLC analysis

N-oxygenation of benzydamine by the wild type and N61S variant of hFMO3 were carried out as previously described [24, 25] and the amount of product determined by HPLC (Agilent-1200, Agilent Technologies, U.S.A.). Each sample was analyzed by HPLC equipped with 4.6x150 mm 5 µm Eclipse XDB-C18 column at room temperature with the UV–visible detector set at 308 nm for benzydamine N-oxide as described previously [25].
2.4 Calculation of the percentage of uncoupling

Uncoupling percentages were calculated by dividing the absolute rates of the rates of benzydamine oxidation by the NADPH consumption which yields the coupled reaction. The uncoupled reaction is given by the subtraction of the coupled reaction from the total reaction (including both coupled and uncoupled reactions).

2.5 Differential scanning calorimetry

In order to provide direct effects of hydrogen peroxide exposure differential scanning calorimetry experiments were carried out for hFMO3 following incubation of the enzyme with hydrogen peroxide. Differential scanning calorimetry was carried out as previously illustrated [13].

2.6 Statistical analyses

Statistical analyses were performed using Sigmaplot 11.0 software. Data calculations were carried out by repeated-measures of two-way ANOVA followed by Student-Newman-Keuls post hoc test. All experiments were executed in triplicates. Data are shown as mean ± standard deviation. Differences among data points were considered significant when p < 0.05.

3. Results

In order to measure the uncoupling reactions of hFMO3, the NADPH oxidation activity of the purified WT enzyme was investigated by monitoring the kinetics of the decrease in absorbance of the reduced cofactor at 340 nm in time. NADPH by itself shows minimal oxidation in the absence of the enzyme, so the reaction can actually start only when both NADPH and hFMO3
are present (data not shown). The amount of $\text{H}_2\text{O}_2$ produced was measured by mixing aliquots of each reaction, at different times after the start, with the AMPLEX red kit components [23]. We compared the $\text{H}_2\text{O}_2$ produced by WT hFMO3 and its polymorphic variant, N61S. The latter, an active site mutant associated with trimethylaminuria is known to have impaired NADP$^+$ binding and lower activity [13]. The data obtained demonstrate how in the absence of the substrate benzydamine (BZD), N61S produces higher amounts of $\text{H}_2\text{O}_2$ compared to WT (Fig 1). Interestingly WT showed steadily increased leakage while N61S had high leakage at the beginning of the incubation that decreased slowly after the first 15 minutes.

![Graph A](image)

**Fig. 1. H$_2$O$_2$ generation for WT and N61S hFMO3.** The amount of hydrogen peroxide was measured for WT (black) and N61S mutant (red) hFMO3 as a function of time after the reaction is started. A) Generation of hydrogen peroxide in the first 5 minutes. B) Generation of hydrogen peroxide for 60 minutes. Reaction conditions for the production of hydrogen peroxide: 1.6 $\mu$M hFMO3 with 160 $\mu$M NADPH at 37°C in the dark in 50 mM KPi at pH 7.4. For detection of hydrogen peroxide refer to Materials and Methods.

If we look at the overall rate of $\text{H}_2\text{O}_2$ production by hFMO3 (Fig 2) the data are in agreement with recently published data [16] for this enzyme (0.5–2.5 nmol/min/nmol FMO at pH 7.4).
**Fig. 2. Rate of H$_2$O$_2$ formation.** Calculation of the rate is performed using the linear range of the kinetics curve between 60 and 180 seconds. Reaction conditions for the production of hydrogen peroxide: 1.6 µM hFMO3 and 160 µM NADPH at 37°C in the dark in 50 mM KPi, pH 7.4.

Moreover, in order to better understand what is the real level of uncoupling in hFMO3, we also measured the amount of product (benzydamine N-oxide) formed as a function of NADPH consumed. As expected the WT enzyme shows overall lower uncoupling (50-70%) when compared to N61S (90-95%), as shown in Figure 3.
**Fig. 3. Enzyme coupling efficiency.** A) Oxidation of NADPH B) Product formation measured as benzydamine N-oxide C) Ratio between the amount of benzydamine N-oxide formed and the amount of NADPH consumed for WT (black) and N61S mutant (red) hFMO3. Reaction conditions for the production of hydrogen peroxide: 1.6 µM hFMO3, 160 µM NADPH with 300 µM benzydamine at 37°C in the dark in 50 mM KPi at pH 7.4.

Further characterization of the uncoupling process was carried out by measuring H$_2$O$_2$ production under different conditions to assess the specific contribution of the substrate benzydamine (BZD), in the presence of catalase (CAT) and superoxide dismutase (SOD). For both WT and N61S enzymes the presence of substrate significantly lowers the formation of H$_2$O$_2$ (Figure 4A, B, Figure S6). As expected, catalase totally removed the H$_2$O$_2$ from the reaction mixtures, so no H$_2$O$_2$ was detected (data not shown). On the other hand, superoxide dismutase in the absence of substrate, increased the amount of H$_2$O$_2$ detected after 15 and 30 minutes suggesting the possible formation of superoxide radical (i.e. since superoxide dismutase catalyzes the disproportionation of superoxide into O$_2$ and H$_2$O$_2$, an increase in H$_2$O$_2$ is indicative of the presence of the superoxide radical) (Figure 4A).
Fig. 4. Effect of substrate, catalase (CAT) and superoxide dismutase (SOD) on H$_2$O$_2$ generation. Data obtained WT (top) and N61S (bottom) hFMO3 after 5 (black), 15 (red) or 30 (blue) minutes. Bars represent the mean ± standard deviation. Statistically different (*) from same time of different group, p < 0.05. Reaction conditions: 1.6 µM hFMO3, 160 µM NADPH, 2 µM SOD with 2 µM catalase at 37°C in the dark in 50 mM KPi at pH 7.4.

Subsequently, the amount of benzydamine N-oxide product formed in the absence or presence of catalase/superoxide dismutase was also calculated. For WT both catalase and superoxide dismutase have a slightly negative impact after 15 minutes on the amount of product formed (Figure 5A). In the case of N61S variant, catalase has a positive impact on catalysis after 15 minutes whereas superoxide dismutase does not seem to affect product formation.
Fig. 5. Effect of substrate, catalase (CAT) and superoxide dismutase (SOD) on benzydamine N-oxide product formation. Data obtained WT (top) and N61S (bottom) hFMO3 after 5 (black), 15 (red) or 30 (blue) minutes. Bars represent the mean ± standard deviation. Statistically different (*) from same time of different groups, *p < 0.05. Reaction conditions: 1.6 μM hFMO3, 160 μM NADPH, 300 μM benzydamine, 2 μM SOD and 2 μM catalase at 37°C in the dark in 50 mM KPi at pH 7.4.

Given the above measured contribution of superoxide dismutase to \( \text{H}_2\text{O}_2 \) production and to clarify whether hFMO3 can actually form the superoxide radical, a cytochrome c reduction assay was carried out [17]. The control reaction was performed using superoxide dismutase to
eliminate any source of superoxide (data not shown). As shown in Figure 6, cytochrome c reduction clearly shows how both WT and N61S hFMO3 are able to form the superoxide radical and that N61S forms twice as much superoxide reaching 4 µM after 5 minutes.

**Fig. 6. Generation of superoxide.** WT (black) and N61S mutant (red) hFMO3. A) Cytochrome c reduction in the absence of benzydamine. B) Cytochrome c reduction in the presence of benzydamine. Reaction conditions: 1.6 µM hFMO3, 160 µM NADPH, 300 µM benzydamine, 10 µM cytochrome c with 2 µM SOD at 37°C in the dark in 50 mM KPi at pH 7.4.

Finally, taken all the data together the total amount of uncoupling measured for both hFMO3 and its N61S polymorphic variant was converted into percentages (see Materials and methods section). In the case of the WT enzyme, after 10 min of incubation with the substrate, ~62% uncoupling was calculated of which 48% is due to hydrogen peroxide with remaining 14% due to superoxide radical formation. On the other hand, under the same experimental conditions, the N61S polymorphic variant is ~98% uncoupled with 80% from hydrogen peroxide and 18% from superoxide formation.
4. Discussion

Mechanistic studies carried out on pig liver FMO during the 1980’s pointed unequivocally towards a precise scheme: mammalian FMO is reduced by NADPH and upon binding of oxygen can form a stable C4a-hydroperoxide intermediate [11,12]. It has been postulated that FMO is present in the cell in this highly reactive form ready to exert catalysis on its substrates [27, 28]. Such mechanism was also investigated against a large number of substrates and even though the enzyme shows differential affinity for N- or S- soft nucleophiles, it is thought to lack a proper binding step during catalysis. According to this scheme, any suitable molecule can be monooxygenated by FMO, provided that charge and size characteristics are respected [27].

In this context, the uncoupling reactions of FMO and its ability to generate reactive oxygen species has been considered negligible for a long time. Nevertheless, more recent studies have highlighted the fact that hFMO3 does not form a highly stable C4a-hydroperoxy intermediate [13]. UV-vis stopped-flow experiments demonstrated that WT hFMO3 forms an intermediate that can last for minutes and not for hours [13, 14]. Further characterization of the enzyme led to the confirmation that NADP+ binding is crucial for the intermediate stability and that an active site mutant, N61S, dramatically decreases the affinity of the enzyme for the cofactor preventing the formation of the reaction intermediate [13, 14]. Measured $K_d$ values for NADPH are 0.3 and 51.8 µM for WT and N61S hFMO3, respectively [13]. In the case of NADP+ the binding affinity for WT is 3.7 µM, whereas for N61S no appreciable binding could be detected [13].

Other published studies on hFMO5 have also pointed towards the absence of a stable intermediate in the reaction mechanism of the enzyme and suggested the unproductive leakage of the peroxyflavin [15]. In a recent published work by Williams and colleagues [16] the uncoupling process of human FMO isoforms 1, 2 and 3 was studied. The latter authors
demonstrated how all studied isoforms generate H$_2$O$_2$ upon reduction by NADPH, both in the presence or absence of substrate and, unexpectedly that the presence of substrate even increased the amount of H$_2$O$_2$ generated by FMO [16]. The same group did not observe any effect with the addition of catalase or superoxide dismutase to the reaction [16].

In this work, we undertook an extensive characterization of hFMO3 on the basis of the above-mentioned recent findings. For this reason, two purified enzymes were used: wild type hFMO3 and N61S [13]. It was found that both WT and N61S produce H$_2$O$_2$ (Figure 1) with higher amounts observed with the mutant due to its inability to bind NADP$^+$ and form a stable C4a-hydroperoxy intermediate. The latter finding suggests that this mutation leads to an overall faster catalytic cycle that is detrimental for catalysis [13]. This is fully in line with previous characterization of the rates of formation and decay of the flavin intermediates reported for WT and N61S hFMO3 [13].

Subsequently, we focused our attention to the actual meaning of coupling i.e. the use of electrons donated by NADPH to yield a product molecule. To this end, benzydamine a hFMO3 marker substrate was used and the amount of product was measured as a percentage of the NADPH consumed. The data confirmed a high degree of uncoupling for hFMO3 and an even higher increased propensity to the unproductive leakage of electrons for the N61S variant (Figure 3). More importantly, if the amount of H$_2$O$_2$ produced in the absence/presence of the substrate is compared, it becomes evident that the presence of the substrate results in a strong decrease in the amount of H$_2$O$_2$ (Figure 4A, B). The latter observation is actually in line with the current representation of the catalytic cycle [16].

All these findings indicate that there are competing paths that ultimately lead to the regeneration of the FAD cofactor: the productive path leads to product oxidation with the unproductive path leading to the formation of reactive oxygen species. Further analysis shows that both catalase
and superoxide dismutase slightly decrease the amount of benzydamine N-oxide formed in the 
enzymatic reaction for WT and have contrasting effects for N61S (Figure 5A, B). A possible 
explanation for the increase in product formation for WT is that N-oxidation can be achieved - 
at least to a much lower extent - in the absence of enzyme and only by H$_2$O$_2$, as most N-oxides 
are actually synthetized [29]. Nevertheless, our experiments show that incubation of H$_2$O$_2$ with 
benzydamine does not lead to non-enzymatic product formation (Figure S7). In the case of 
N61S, a significant increase in the amount of product is detected after 30 minutes in presence 
of catalase. This could be due to the decreased protein structural damage caused by large 
amounts of H$_2$O$_2$ in the absence of catalase after several minutes/catalytic cycles. Our data 
indicate that incubation of H$_2$O$_2$ with hFMO3 does not lead to structural damage (Figure S8). 
Therefore the increased amount of product formed for N61S can only be compatible with a 
higher coupling efficiency in the presence of catalase.

In general, looking at the catalytic cycle of hFMO3 (Fig. 7) a productive oxidative half-cycle 
leading to N-oxidation of the substrate and an unproductive oxidative half-cycle leading to H$_2$O$_2$ 
can clearly be identified. As expected in the presence of the substrate the enzyme uses the 
productive path more often as confirmed by data obtained in presence of benzydamine showing 
that the presence of this substrate favors the coupled reaction and decreases the amount of 
H$_2$O$_2$ formed. Moreover, since a higher amount of H$_2$O$_2$ was observed in the presence of 
superoxide dismutase, the possibility of hFMO3 producing the superoxide radical was also 
investigated. Cytochrome c reduction experiments confirmed hFMO3 as the source of 
superoxide radical. As mentioned earlier, superoxide radical formation had already been 
reported for pig FMO but it had been studied in close association to the possible exploitation of 
this reactive oxygen species for the hydroxylation of amines [17] and not in the context of the 
uncoupling reactions.
In light of the observed superoxide radical, we propose a second shunt in the catalytic cycle of hFMO3 consisting of an earlier exit from the productive path (fig. 7, steps a, b). The latter is in addition to the already reported shunt leading to \( \text{H}_2\text{O}_2 \) (fig. 7, step c) as mentioned above [16]. It is known that for the reduced flavin to react with oxygen a “radical pair” between the superoxide and the flavin semiquinone is formed (fig. 7 step 2) [30]. This species is highly unstable and it has never been captured [31], but it is chemically required to bypass the spin inversion barrier [30, 31]. At this point of the cycle the radical pair can either yield the hydroperoxyflavin (fig. 7 step 3-4) or take an unproductive path and generate a semiquinone radical with the loss of the superoxide (fig. 7 step a). This semiquinone intermediate can decay resulting in the re-oxidized flavin with the concomitant production of another superoxide (fig. 7 step b). This new path can explain why after the formation of the caged radical pair, superoxide can detach from the flavin together with NADP\(^+\), short circuiting the catalytic cycle.
Fig. 7. Chemical structures of FAD reaction intermediates during the catalytic cycle of flavin-containing monooxygenases. NADPH reduction (step 1) is rapidly followed by activation of molecular oxygen (step 2), binding on the C4a and protonation to yield the C4a-hydroperoxide (step 3-4). This intermediate can react with the substrate (S) and yield the oxygenated product (S-O, step 5). The second atom of oxygen is released as H$_2$O (step 6) and NADP$^+$ can leave the active site (step 7). Two side reactions can occur that produce reactive oxygen species: steps a) and b) before formation of the hydroperoxyflavin after spin inversion of the radical pair which can result in the loss of superoxide or step c) the direct loss of H$_2$O$_2$ from the C4a-hydroperoxy intermediate.
The physiological role of the uncoupled reaction products of hFMO3 is still unknown. It has been suggested that the generation of hydrogen peroxide by FMO could play a role in control of the overall redox state of the cell [27] or in the synthesis of protein disulfide bonds through cysteamine oxidation [32, 33]. On the other hand, toxicological effects such as hepatic injury through radical production and lipid peroxidation have also been reported in rat FMO catalyzing the oxidation of thioacetamide [34]. Initially it was thought that the products of thioacetamide, being radicals themselves, were initiating the inflammation and the resulting hepatic injury, but further investigation suggested the FMO enzyme itself might be involved [35]. Nevertheless, since this current work and others [16-18] have shown FMO enzymes to be highly uncoupled, further studies are required to confirm whatever the role of these reactive oxygen species maybe, physiological or toxicological.

Conflict of interest
The authors declare no conflict of interest

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